Non-canonical modulation of cellular signaling by the viral chemokine receptor ORF74

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Chapter 6

Conclusions and discussion

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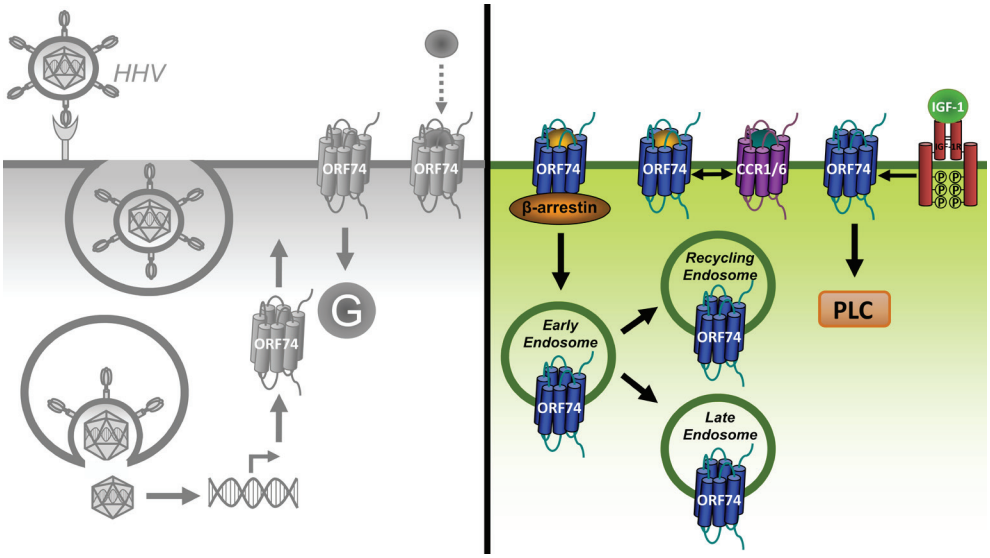

The aim of the research described in this thesis was to investigate the mechanisms employed by KSHV-encoded ORF74 to redirect cellular signaling. As described in the introduction, canonical mechanisms can be distinguished from non-canonical mechanisms:

1. Hijacking human chemokines by ORF74
2. Hijacking human G proteins by ORF74

} Canonical mechanism

3. Hijacking the human endocytic machinery by ORF74 (**Chapter 2**)
4. Hijacking human GPCRs by ORF74 (**Chapter 3**)
5. Hijacking human RTKs by ORF74 (**Chapter 4**)

} Non-canonical mechanism

Although the canonical mechanisms have been the subject of most studies that focused on ORF74, they are still not fully understood. Questions that remain to be answered are discussed in the first part of this final chapter. However, the more recently recognized non-canonical mechanisms are the subject of this thesis and will be discussed in the second and largest part of this chapter. Each subsection of the non-canonical mechanisms starts with a figure summarizing the major findings, followed by a discussion on the implications of these findings. How do these findings compare to other human and viral GPCRs and how do they contribute to the general knowledge and models available in the field? It is speculated how hijacking of cellular signaling via non-canonical mechanisms might be beneficial for the virus. Furthermore, limitations of the used research methods are critically reviewed. Finally, it is discussed how the findings described in this thesis might contribute to the development of new therapeutics that target these non-canonical mechanisms of ORF74 and we will reflect on the challenges of identifying specific small-molecule inhibitors, as were encountered in **Chapter 5**.

1. HIJACKING OF HUMAN CHEMOKINES

Information about the structural basis for chemokine binding to ORF74 is scarce (~3 publications) [153, 154, 235] and mainly originates from mutagenesis studies. The exact binding mode of the different chemokines is unclear and it remains to be elucidated how ORF74, in contrast to its human counterparts, can interact with chemokines from different classes (CC and CXC). Furthermore, the exact role of chemokine-induced signaling in comparison to constitutive signaling is not fully investigated. Insight in how and why chemokines bind to ORF74 might aid in developing compounds that inhibit chemokine binding and chemokine-induced signaling. These inhibitors might represent potential therapeutics for the treatment of KSHV-associated pathologies and research tools for elucidating the role of chemokine binding.

1.1 How do chemokines bind to ORF74 (and chemokine receptors in general)?

Like most human chemokine receptors [490], truncation of the N-terminus of ORF74 abolishes chemokine binding. Removing the first 11 amino acids reduced the binding affinity for CXCL10 but retained high affinity binding to CXCL1, whereas removing the first 22 amino ac-

ids completely inhibited CXCL1 and CXCL10 binding [153] (Fig. 1). Furthermore, only CXCL1 binding is dependent on sulfation of Y^{26} and Y^{28} of ORF74 [154] (Fig. 1), suggesting that CXCL1 and CXCL10 differentially bind to the N-terminus of ORF74. Tyrosine sulfation is also important for chemokine binding to most human chemokine receptors [491]. Interestingly, CXCL8 binds to a much smaller population of ORF74 as compared to CXCL1 and CXCL10 [367], which might indicate that the N-terminus alone is not sufficient for CXCL8 binding. Indeed, a C-terminal deletion mutant of ORF74 is unable to bind CXCL8, but retained the ability to bind CXCL1. This suggests that helix 8 stabilizes a conformation of TM7 that is crucial for CXCL8 binding, but not for CXCL1 binding [235].

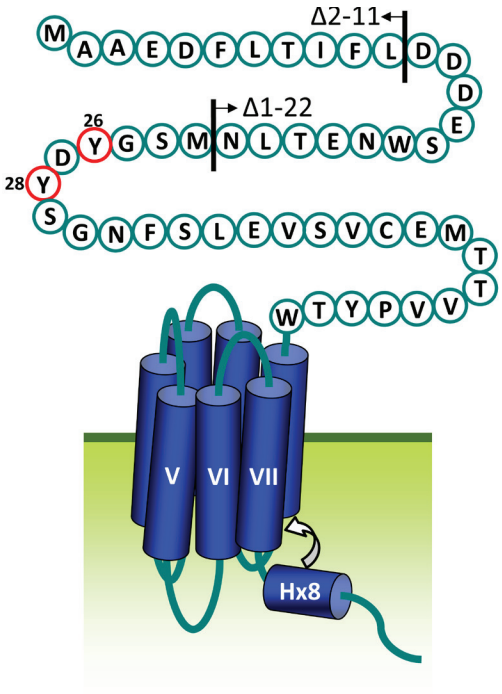

Figure 1. Molecular determinants for chemokine binding to ORF74. *N-terminal deletion mutants (Δ2-11 and Δ1-22) show that the first 11 amino acids play a role in CXCL10 binding, whereas the first 22 amino acids are key for both CXCL1 and CXCL10 binding. Two tyrosine residues in the N-terminus (red) are crucial for the binding of CXCL1, but not CXCL10. Helix 8 is involved in CXCL8 binding, probably by stabilizing TM7 (white arrow).*

More information on the binding mode of chemokines to their receptors became available with the recently published crystal structures of CXCR4 in complex with KSHV-encoded vCCL2 [492] and US28 in complex with CX3CL1 [161]. In both structures, the N-terminus and ECL2 of the receptor interacts with the globular body of the chemokine, whereas the N-terminus of the chemokine projects into the TM domain of the receptor (Fig. 2). This binding mode is consistent with earlier performed mutagenesis, binding and activation studies on US28 [160], ORF74 [153] and several other chemokine and chemokine receptors, which had led to the general concept of chemokine binding as described in the two-site binding model [152, 162]. For example, studies investigating the interaction of chemokines with peptides corresponding to the N-termini of chemokine receptors (e.g. CCR3, CXCR1 and CXCR3) showed the importance of receptor N-termini for chemokine binding [490]. Furthermore, N-terminal truncated chemokine mutants such as CCL2, CCL4, CCL5, CCL14 and CXCL11 are

unable to activate their respective receptor while retaining binding affinity [493-496], suggesting that the N-termini of chemokines are important for receptor activation. It would be interesting to see whether the N-termini of CXCL1 and CXCL10 differentially interact with the TM domain of ORF74, which could potentially explain why these chemokines act as an agonist and inverse agonist, respectively.

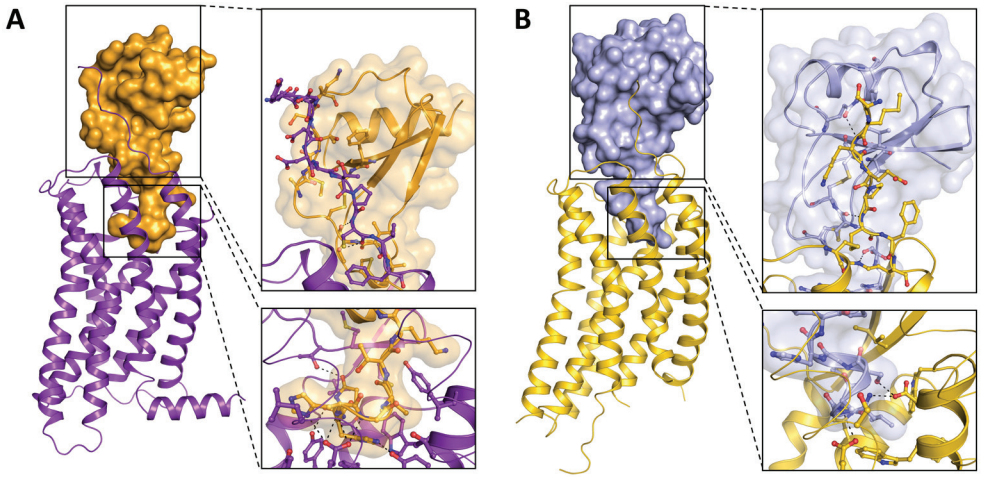

***Figure 2. Crystal structures of US28 (A) and CXCR4 (B) in complex with a chemokine**. The N-terminus of US28 and CXCR4 bind to the globular body of CX3CL1 and vCCL2, respectively, whereas the N-terminus of the chemokine interacts with the receptor TM pocket. Image made by Albert J. Kooistra.*

Interestingly, modeling of a complex between CXCR4 and its endogenous ligand CXCL12 and between CCR5 and vCCL2 revealed conformational differences between bound CC and CXC chemokines. In combination with the identification of highly conserved residues specifically present in CC or CXC chemokine receptors, this might explain the specificity of CC and CXC chemokine recognition by chemokine receptors [492]. CXCR4 possesses features that are typically found in CXC chemokine receptors but also in CC chemokine receptors, such as a basic residue at position C+2 (where C is the conserved cysteine in the N-terminus) and the presence of a sulfotyrosine in the proximal N-terminus of CXCR4 (position C-7). This might explain why CXCR4 not only binds CXCL12, but is able to bind the CC chemokine vCCL2 [492]. Interestingly, ORF74 contains an acidic residue at position 6.58 (typical for CXC chemokine receptors) but none of the typical CC chemokine receptor features. As such, it remains to be investigated how ORF74 is able to bind CC chemokines such as vCCL2. Crystal structures of ORF74 in complex with different chemokines might provide answers.

1.2 Why should ORF74 bind chemokines?

ORF74 shows highest sequence identity to CXCR2 and appears to be pirated from the host genome during co-evolution. Over time, ORF74 (and other vGPCRs such as US28 and U51) has acquired the ability to signal in the absence of ligands (i.e. constitutive activity), but retained the ability to bind chemokines. This suggests that chemokine binding provides some advantage for KSHV. On the other hand, this might not hold true for all vGPCRs, as some of them are orphan receptors (e.g. BILF1 and UL33).

One hypothesis is that chemokines such as CXCL1 are required to increase the basal signaling of ORF74 to reach a certain threshold. This might explain why constitutive activity and chemokine-induced signaling are both required for the development of KS-like lesions in a mouse model [155].

Another possibility is that chemokines activate specific signaling cascades that are not activated by the constitutive activity of ORF74. Indeed, CXCL1 is necessary for the ORF74-induced transcriptional activation of the lytic switch ORF50 in the PEL cell line BC3.14 [497] (but not in HEK293T cells [498]). Furthermore, in **Chapter 4** it is shown that ORF74 recruits β-arrestins only in response to chemokines. In a similar fashion, chemokines are required for US28-, HHV7-U12- and HHV6-U51-mediated Ca^{2+} mobilization and cell migration [130, 171, 204, 222]. ORF74-mediated Ca^{2+} signaling and cell migration in response to different chemokines have not been investigated yet.

CXCL8 modulates ORF74 signaling and promotes β-arrestin recruitment to ORF74 at concentrations (> 100 nM) much higher than its potency to activate the human chemokine receptor CXCR2 (EC_{50} = 3 nM [499]). Systemic CXCL8 levels in the blood are generally much lower (pM range) [500, 501] as compared to the CXCL8 concentrations required to modulate constitutive ORF74 activity, but might vary locally during inflammation [502-504]. As such, the physiologically relevance of CXCL8-mediated ORF74 signaling remains to be elucidated. It is unlikely that CXCL8 functions to antagonize CXCL1- and CXCL10-mediated signaling, as CXCL1 and CXCL10 bind to ORF74 populations that are largely unavailable for CXCL8 (see also section 1.1) [367]. Possibly, CXCL8 acts as a high-potency agonist for yet undiscovered (non-canonical) ORF74 signaling pathways, such as the transactivation of RTKs. Besides chemokines that bind to CXCR2, ORF74 also acquired the ability to bind the CXCR3 and CXCR4 chemokines inverse agonists CXCL10 and CXCL12, respectively, suggesting that the negative regulation of ORF74 is also beneficial for KSHV (see also section 3.2.2.1).

2. HIJACKING OF G PROTEINS

ORF74 is often described to promiscuously bind to $G\alpha_{i/o}$, $G\alpha_{12/13}$ and $G\alpha_{q/11}$ [50]. However, these conclusions are mainly based on the overexpression of (constitutive active mutants of) Gα subunits [179, 180] or inhibitors acting on targets downstream of G proteins. For example, ORF74 is assumed to activate Akt, ERK and the ORF50 promoter in a $G\alpha_q$-dependent manner based on the effect of a phospholipase C (PLC) inhibitor [181, 498], but direct evidence showing that ORF74 activates PLC via $G\alpha_q$ is lacking. Experiments where G proteins are selectively removed from the system, as in $G\alpha_q$-deficient mouse embryonic fibroblast (MEF) cells that were used to show $G\alpha_q$-dependent PLC activation mediated by US28 [285], have not been performed for ORF74. Furthermore, contradicting results with respect to the role of $G\alpha_i$ in CRE activation have been reported for ORF74. Whereas PTX had no effect on ORF74-mediated CRE activation in HEK293 cells [205], ORF74 activates CRE in a PTX-sensitive manner in the PEL cell line BC3.14 [179].

To obtain more insight in G protein-dependent signaling of ORF74, we attempted to study selective G protein coupling by means of a previously described yeast-based assay [505-

507]. Ten different yeast strains, each expressing the individual human Gα protein subtypes as chimeric fusion with the yeast Gα protein Gpa1, enable the functional coupling of mammalian (or viral) GPCRs to the yeast pheromone response pathway that promotes cell growth [508]. Unfortunately, we were unable to detect ORF74 expression in yeast (data not shown) and the G protein subtypes activated by ORF74 remain to be identified.

3. HIJACKING OF β-ARRESTINS

The aim of the research described in **Chapter 2** was to investigate whether ORF74 hijacks proteins involved in endocytic trafficking and our data (Fig. 3) show that:

- ORF74 recruits β-arrestin1/2 in response to human chemokines. The residues S^{335}, S^{338}, T^{241} and T^{242} in the C-tail of ORF74 were identified as the molecular determinants for β-arrestin recruitment by site-directed mutagenesis studies and is in line with homology modeling. This suggests that the phosphorylation of the C-tail of ORF74 is essential for β-arrestin recruitment.

- ORF74 internalizes and traffics via early endosomes to recycling and late endosomes in a β-arrestin-dependent manner

3.1 Molecular determinants for the interaction of β-arrestins with GPCRs

Arrestins form a small family of proteins with only four members in mammals, of which two members (β-arrestin1 and β-arrestin2) regulate the majority of GPCRs. A lot of effort has been put into elucidating the receptor-binding determinants to understand how only two β-arrestin proteins interact with hundreds of different GPCRs [354].

3.1.1 Multiple phosphorylation sites

Similar to ORF74, the majority of human GPCRs depend on phosphorylated serine and threonine residues in their C-tail (and sometimes intracellular loops) for high affinity interaction with β-arrestins [509]. These negatively charged phosphates interact with positively charged lysines and arginines of β-arrestin [343]. Site-directed mutation of only the distal serine residues or threonine residues in the C-tail of ORF74 is enough to abrogate β-arrestin recruitment in response to CXCL1, suggesting that multiple phosphorylation sites are required. Likewise, at least two phosphates are required for arrestin1 binding to rhodopsin and maximum arrestin1 binding is observed with four phosphates [510]. Similarly, CCR5 mutants with Ala-substitution of any two of the four serine residues in their C-tail that are phosphorylated in response to CCL5, retain their ability to recruit β-arrestins. However, Ala-substitution of any three of these four serine residues completely abolished β-arrestin recruitment [358]. Interestingly, the C-tail of ORF74 encoded by Saimirline herpesvirus 2 (SHV2) only contains a single threonine residue and it remains to be investigated whether this receptor is able to recruit β-arrestins.

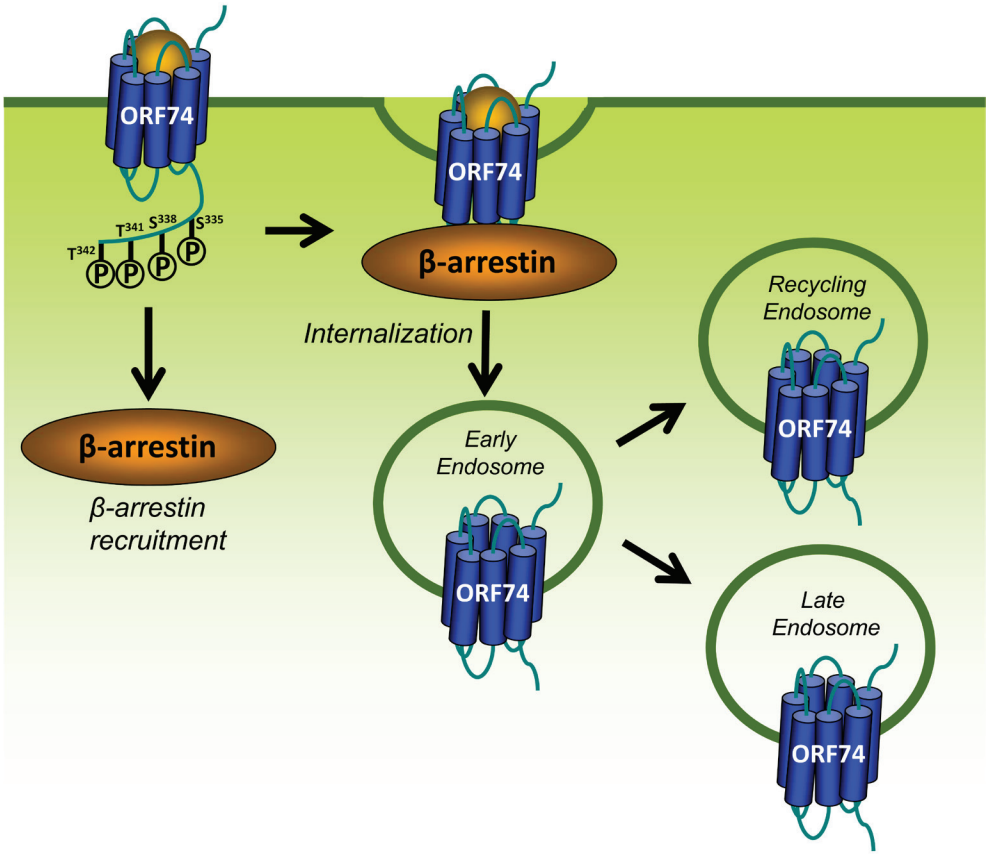

Figure 3. ORF74 hijacks human β-arrestin-dependent endocytosis-routes. *Distal S/T residues in the C-tail of ORF74 are required for β-arrestin recruitment and subsequent receptor internalization and trafficking to endosomes (early, recycling and late endosomes).*

In contrast, some receptors recruit β-arrestin in a phosphorylation-independent manner [352, 511-513]. Possibly, negatively charged aspartic acid and glutamic acid residues act as phospho-mimics and engage in the charge-charge interaction with positively charged residues of β-arrestin [512, 514].

3.1.2 Model of the β-arrestin-GPCR interaction; what we know and do not know

Besides phosphorylated S/T residues, GPCRs also need to adopt an active conformation for high affinity β-arrestin binding and the current model for the β-arrestin-GPCRs interaction describes a bimodal interaction, consisting of a 'phosphorylation sensor' and an 'activation sensor' that act in a synergistic fashion [509]. The phosphorylation sensor consists of two major sets of intramolecular interactions involving the C-tail of β-arrestin that constrain β-arrestin in an inactive conformation [515]. Upon binding to a GPCR, the C-tail of β-arrestin is displaced by the phosphorylated C-tail of the GPCR, exposing binding sites for proteins from the endocytotic machinery (e.g. clathrin) located on the C-tail of β-arrestin. Displacement of the β-arrestin C-tail disrupts the intramolecular constrains and β-arrestin

consequently adopts an active conformation [516]. This conformational change allows the activation sensor of β-arrestin (i.e. the 'finger loop', which adopts an extended conformation upon β-arrestin activation) to interact with the cavity between the TM helices of GPCRs that is opened during GPCR activation [509]. The bimodal interaction and conformational changes of β-arrestin upon activation are consistent with a recently proposed model of the β_2 adrenergic receptor (β_2AR)–β-arrestin-1 complex based on electron microscopy and deuterium exchange mass spectrometry [517] and recently released crystal structures of active β-arrestin1 bound to the phosphorylated C-tail of the Vasopressin 2 receptor (V_2R) [343], a C-tail truncation mutant of active arrestin-1 [518] and rhodopsin in complex with a peptide analogue of the finger loop of arrestin-1 [519]. Interestingly, the latter shows a direct interaction between the finger loop of arrestin-1 and $R^{3.50}$ of the DRY motif of rhodopsin. On the contrary, ORF74-$R^{3.50}$A is able to recruit β-arrestin1/2 with a potency and efficacy comparable to WT-ORF74 (see **Chapter 2**), indicating that $R^{3.50}$ of ORF74 is not essential for the interaction with β-arrestin1/2.

What remains unclear after the release of the β-arrestin1/V_2Rpp crystal structure is how β-arrestins interact with GPCRs that have a distinct number and spatial distribution of S/T residues as compared to V_2R. ORF74 shows spatial conservation of S/T residues but, the C-tail of β_2AR is much longer than the C-tail of V_2R (and ORF74) and contains many S/T residues that do not align with the S/T residues in the V_2R C-tail that interact with β-arrestin1. Interestingly, it has been suggested that these β_2AR S/T residues might be differentially phosphorylated by GRK2 and GRK6 to establish specific phosphorylation patterns or 'phosphorylation barcodes' that determine β-arrestin's downstream effects. GRK2 and GRK6 promote β-arrestin2 to adopt distinct conformations, as was shown by an intramolecular BRET-based biosensor. As a consequence, GRK2-mediated phosphorylation of β_2AR regulates internalization whereas GRK6-mediated phosphorylation is involved in β-arrestin-dependent ERK phosphorylation [520]. This would suggest that β-arrestins could have multiple binding sites located on the C-tail of GPCRs. The relatively short C-tail of ORF74 might not be able to accommodate these different β-arrestin binding sites. However, it can not be excluded that only a subset of the four identified S/T residues within the β-arrestin binding motif in the C-tail of ORF74 needs to be phosphorylated for high affinity binding to β-arrestin. Hence, these four S/T residues might be differentially phosphorylated by distinct GRKs to establish specific phosphorylation barcodes. Over-expression of GRK5 and GRK6, but not GRK2, inhibit constitutive PLC activation mediated by ORF74 [278]. However, it remains to be elucidated which GRKs are involved in β-arrestin recruitment and internalization of ORF74.

3.2 Endocytic trafficking

3.2.1 Two internalization mechanisms for ORF74?

The β2-subunit of the clathrin adaptor protein AP-2 binds to the C-tail of GPCR-bound β-arrestins and regulates clathrin-mediated internalization of many GPCRs, including β_2AR and V_2R [521]. However, AP-2 is also able to directly interact with a tyrosine residue in the C-tail of PAR-1 via its μ2-subunit and is essential for constitutive PAR-1 internalization [264]. Likewise, Y^{326} in the C-tail of ORF74 was recently identified to constitutively interact with AP-2 [189]. Indeed, Ala-substitution of this tyrosine residue disrupts the interaction with AP-2 and inhibits constitutive ORF74 internalization [189]. Hence, ORF74 contains two inter-

nalization-determinants in its C-tail: Y^{326} and S/T residues for AP-2-mediated and β-arrestin-dependent internalization, respectively.

Possibly, constitutive internalization of ORF74 occurs via Y^{326} and AP-2, whereas ligand-induced internalization is regulated by S/T residues and β-arrestin. Indeed, the lack of constitutive β-arrestin recruitment (see **Chapter 2**) supports this model. Distinct internalization mechanisms for constitutive versus ligand-induced internalization have been reported for several GPCRs [522, 523]. For example, ligand-induced internalization of GPR40 is β-arrestin, GRK2 and clathrin dependent, whereas constitutive GPR40 internalization is not affected by knockdown of these proteins [524].

Alternatively, AP-2 might bind simultaneously to Y^{326} and ORF74-bound β-arrestin with its μ2- and β2-subunits, respectively, to establish the high-affinity binding to ORF74. Eliminating only one of these AP-2 binding sites would be enough to inhibit ORF74 internalization. This dual interaction of AP-2 with both β-arrestin and receptor was proposed to underlie the internalization of the calcium sensing receptor CaSR (a class C GPCR that binds Ca^{2+}) [525].

Investigating whether ORF74-Y^{326}A could recruit β-arrestin and internalizes in response to chemokines or whether ORF74-ST/A2 is still able to interact with AP-2 would shed light on these two proposed models.

3.2.2 Relevance of endocytic trafficking of ORF74

Our finding that constitutively active ORF74 is removed from the host cell surface by endocytosis, suggests that it is somehow beneficial in the viral context.

3.2.2.1 Balancing signaling

The basic principle of GPCR desensitization and internalization is to regulate the magnitude and duration of GPCR signaling and subsequently to protect cells from overstimulation. Dysregulated desensitization and/or trafficking of human GPCRs might lead to aberrant signaling and is linked to various diseases [526, 527]. Although a little counterintuitive for a constitutively active GPCR, sustained ORF74 signaling might lead to disruption of cellular homeostasis. KSHV developed different strategies to negatively regulate ORF74 expression and signaling, further supporting that a balanced signaling of ORF74 might be beneficial for KSHV. For example, KSHV-encoded K7 promotes the proteasomal degradation of ORF74 and consequently reduces ORF74 tumorigenicity in nude mice [528]. Moreover, the KSHV-encoded chemokine vCCL2 acts as an inverse agonist for ORF74 [148]. Finally, ORF74 is transcribed as bicistronic mRNA at the 3' end, resulting in inefficient translation and subsequent limited expression [529]. Internalization might be an additional strategy employed by KSHV to regulate temporal ORF74 signaling.

3.2.2.2 Intracellular signaling

Another possibility is that ORF74 internalizes to initiate a second wave of signaling from intracellular vesicles. For example, endosomes (e.g. early, late or recycling endosomes) contain a wide range of signaling molecules incorporated in their membranes (e.g. G proteins and adenylyl cyclase (AC)) and might facilitate GPCR signaling [530]. Data on thyroid-stimulating hormone receptor (TSHR) [531], the parathyroid hormone receptors (PTHR) [532], the sphingosine 1-phosphate 1 receptor (S1P1R) [533] and β_2AR [534-536] led to the hypoth-

esis that internalized GPCRs might signal via G proteins from endosomes to trigger specific downstream effects that are distinct from the signals initiated from the cell membrane.

Besides proteins from the endocytic machinery, β-arrestins scaffold numerous signaling proteins to enhance or inhibit their activities [276]. Hence, ORF74 might signal via β-arrestins in a G protein-independent manner after internalization.

3.2.2.3 Viral assembly

During the last step of viral assembly, the viral envelope is acquired by budding into cellular vesicles. The HCMV envelope contains markers for both the trans-Golgi network (TGN) and endosomes and it is hypothesized that the membranes used by HCMV for its envelope are derived from the transport vesicles between endosomes and the TGN [537]. After synthesis, viral envelope proteins may be transported to these vesicles via the TGN. The HCMV-encoded GPCRs might reach these vesicles via an alternative route, after first being transported to the cell surface to modulate cellular signaling. Indeed, US27, US28, UL33 and UL78 are constitutively internalized from the cell surface and mainly localized in endocytic compartments in HCMV-infected cells [290, 291]. Hence, HCMV may exploit the human endocytic machinery to deliver its vGPCRs to endocytic vesicles and subsequently to the vesicles between endosomes and the TGN where they are incorporated into the viral membranes during the final stages of virus assembly. Indeed, the HCMV-encoded GPCRs are found on the membranes of viral particles [123, 290, 538], possibly to allow their deposition on the membrane of infected cells immediately upon infection.

ORF74 is not reported to be present on viral particles. This makes sense as ORF74 expression is tightly regulated and restricted to the lytic phase [337]. Therefore it seems unlikely that ORF74 hijacks the endocytic machinery to be incorporated into the viral membrane.

3.2.2.4 Intracellular hiding

Antibodies form one of the first lines of defense against viral infections [539]. They may recognize viral proteins exposed on the cell surface of infected cells, such as vGPCRs [540]. Hence, internalization removes vGPCRs from the cell surface, preventing their exposure to antibodies and subsequently suppresses the elimination of virus-infected cells [541]. US28 is mainly expressed intracellular due to its constitutive internalization, suggesting that it indeed hides from the immune system. In contrast, ORF74 is mainly expressed on the cell surface [205], but the restricted expression on a small subfraction of cells in the lytic face of infection might limit its exposure.

3.2.2.5 Co-internalization of host immune-related proteins

Internalization of ORF74 might also contribute to immune evasion by downregulating host immune proteins. For example, ORF74 interacts with and mediates the downregulation of the toll-like receptor 4 (TLR4), which plays a key role in the innate immunity against KSHV [281]. The internalization-deficient mutant ORF74-Y^{326}A [189] (see section 3.2.1) is unable to downregulate TLR4 [280]. This might indicate that TLR4 co-internalizes with ORF74. It remains to be investigated if β-arrestin also plays a role in TLR4 downregulation or whether this is specifically regulated via the constitutive interaction with AP-2.

Likewise, BILF1 reduces the cell surface expression of MHC class I proteins (molecules that

display fragments of viral proteins that are recognized by cytotoxic T cells [542]) and subsequently T cell recognition [95, 96]. Although internalization of BILF1 has not been reported, the C-tail of BILF1 is essential for downregulation of MHC class I proteins [95, 96]. Ala-substitution of the single tyrosine residue (Y^{303}) in the C-tail of BILF1 did not alter downregulation of MHC class I proteins [95], which is consistent with the observation that AP-2 is not required for removing MHC class I proteins from the cell surface [96]. Instead, S/T residues in the C-tail of BILF1 and subsequent β-arrestin recruitment might play a role in MHC class I protein downregulation. Indeed, the BILF1 homolog encoded by marmoset LCV lacks several S/T residues in its C-tail as compared with EBV- and rhesus LCV-encoded BILF1 and is unable to downregulate MHC class I proteins. However, replacing the C-tail of marmoset LCV-BILF1 by the C-tail of EBV-BILF1 does not rescue MHC class I downregulation, suggesting that the EBV-BILF1 C-tail is required but not sufficient to regulate MHC class I protein levels [95].

3.2.2.6 Chemokine scavenging

The atypical chemokine receptors ACKR2 (D6), ACKR3 (CXCR7) and ACKR4 (CCX-CKR) bind various chemokines but do not activate typical G protein-dependent signaling or stimulate migration. Instead, these receptors constitutively internalize chemokines and subsequently target them for degradation, resulting in the depletion of extracellular chemokines. For example, pro-inflammatory chemokine scavenging by ACKR2 suppresses the interaction of leukocytes with lympatic endothelial cells and subsequently inhibits inflammatory responses. The relevance of chemokine scavenging in vivo has been confirmed in ACKR2 null mice, which show enhanced inflammation as compared to wild-type mice [543, 544]. Chemokine scavenging by ACKR3 internalization creates a graded distribution of CXCL12 and consequently directs proper cell migration mediated by the CXCR4/CXCL12 axis during embryonic development of zebrafish [545].

Interestingly, US28 was also proposed to act as a chemokine scavenger to limit the attraction of immune cells that induce anti-viral responses. Indeed, CCL2 and CCL5 are depleted from the medium of HCMV-infected cells and US28 inhibits the migration of monocytes [165]. However, US28 was unable to impair the attachment of monocytes to an endothelial monolayer [166], questioning the relevance of chemokine scavenging by US28.

Since only a small subfraction of KSHV-positive cells in PEL and MCD tumors and in KS lesions express ORF74 (0.5-5%) [337], it seems unlikely that ORF74 internalization significantly contributes to the depletion of extracellular chemokine levels to escape anti-viral immune responses. Furthermore, ORF74 promotes the secretion of pro-inflammatory chemokines such as CXCL1 and CXCL8 in ORF74-transfected COS-7 cells and a KS-derived cell line [82, 546], which seems to contradict with a role for ORF74 as chemokine scavenger at least for these chemokines.

4. HIJACKING OF HUMAN GPCRS

The aim of **Chapter 3** was to investigate whether ORF74 heterodimerizes with human chemokine receptors, potentially to modulate their function in favor of KSHV. Our findings are:

- A combination of three different techniques led to the conclusion that ORF74 is in close proximity with CCR1, CCR6 and ORF74 (BRET) on the cell surface (Proximity ligation assay) of transiently transfected HEK293T cells and co-immunoprecipitation demonstrated a direct physical interaction (Fig. 4).

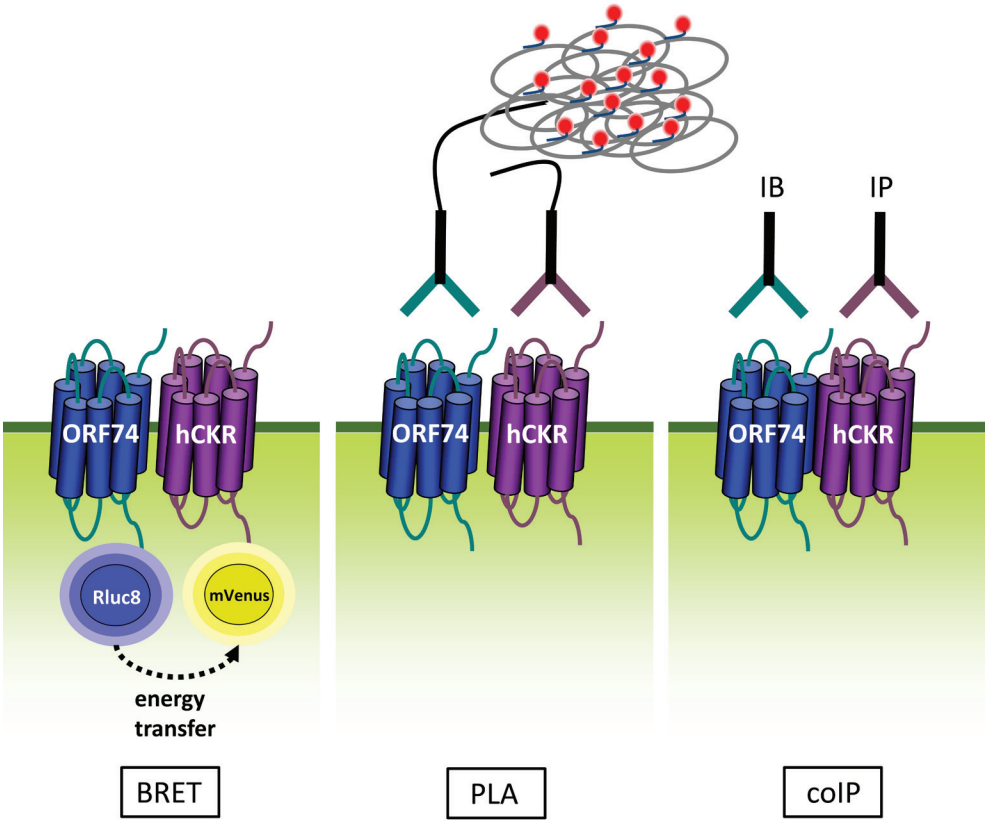

Figure 4. Techniques used to study GPCR dimerization. (left) *ORF74 and human chemokine receptors (hCKR) are C-terminally fused to Rluc8 and mVenus, respectively. Close proximity between ORF74 and hCKRs is reflected by Bioluminescence Resonance Energy Transfer (BRET) between Rluc8 and mVenus.* ***(middle)*** *In the Proximity Ligation Assay (PLA), oligonucleotides form circular DNA with DNA strands attached to ORF74- and hCKR-targeting antibodies. The circular DNA is amplified via the rolling-circle amplification reaction and fluorescent-labeled probes complementary to the amplified DNA allow for the visualization of close proximity between ORF74 and hCKRs.* ***(right)*** *To detect a direct physical interaction between ORF74 and hCKRs, hCKRS are immunoprecipitated (IP). Co-immunoprecipitated ORF74 is visualized by immunoblotting (IB).*

4.1 The physiological relevance of GPCR dimerization

Techniques using recombinant cell systems have been dominated the GPCR dimerization field, but the existence of GPCR dimers in a native context and whole animal models remained largely unknown. The demonstration that rhodopsin and β_2AR could efficiently activate their respective G proteins as monomers [547, 548] further questioned the in vivo relevance of GPCR dimerization. As such, an IUPHAR committee suggested in 2007 that the following criteria should be met in order to define physiologically relevant GPCR dimers [549]:

- Evidence for GPCR dimer in native tissue or primary cells
- A unique functional or biochemical property that can be assigned to the GPCR dimer

As a result, the GPCR dimerization field has now been shifted to identifying dimers in native tissues using techniques such as co-immunoprecipitation [550]. However, co-immunoprecipitation does not distinguish between a direct interaction of GPCRs and GPCRs that co-immunoprecipitate with each other via scaffolding proteins. Alternatively, close proximity of oxytocin receptors in mammary gland tissue of rats was detected by measuring fluorescence resonance energy transfer (FRET) between two fluorescently labeled antagonists [393]. However, this technique would not be applicable to GPCR dimers that display negative ligand binding cooperativity between the protomers [295, 296] or orphan receptors. Other techniques that have been used to demonstrate GPCR dimers in native tissue include atomic force microscopy [551], heterodimer-selective ligands [552] or antibodies [553] and the proximity ligation assay as introduced in **Chapter 3** [397, 554]. GPCR dimerization in vivo was convincingly demonstrated by Huhtaniemi and colleagues. A binding-deficient and signaling-deficient mutant of the luteinizing hormone receptor (LHR) are nonfunctional on their own and transgenic mice expressing these mutants presented similar phenotypes to those observed for LHR knockout animals [555]. However, these LHR mutants functionally complemented each other when co-expressed in transgenic mice and rescued the WT phenotype. This is most likely achieved by the physical interaction of LH receptors [555].

Hitherto, a few studies demonstrated the functional role of GPCR dimerization in a native environment. For example, activation of β_1 and α_{1B} adrenergic receptors results in the production and secretion of melatonin by the pineal gland in the brain of rats. The D_4 receptor, which is expressed in the pineal gland only during dark periods (i.e. night), formed heterodimers with both adrenergic receptor and consequently inhibited β_1 and α_{1B} receptor-mediated melatonin production [397]. Thus the altered expression of D_4 in the pineal gland during day and night cycles allowed for studying the effect of heterodimers in vivo without interfering with the system (e.g. knockout animal).

However, it is difficult to distinguish functional consequences of GPCR heterodimerization from downstream crosstalk between co-expressed GPCRs. Strong evidence to demonstrate that the direct physical interaction of both receptors is required for the altered pharmacological properties involves the disruption of the GPCR dimer. A peptide corresponding to the TM domains of the angiotensin receptor type 1a (AT1aR) and the secretin receptor (SCTR) reduced BRET between these receptors, consistent with a conformational change of the heterodimer. Injecting this peptide into mice suppressed the hyperosmolarity-induced water-drinking behavior, suggesting an important functional role of the AT1aR/SCTR heterodimer in vivo [556]. However, interpretation of these results is challenging as it remains to be seen whether this peptide does not directly affect ligand binding or signaling of the monomeric receptors.

4.2 Does ORF74 form dimers?

The use of three different techniques (BRET, PLA and co-immunoprecipitation) that complement each other and all support the same conclusion, gives confidence that ORF74 indeed physically interacts with human chemokine receptors. However, all data from chapter 3 was

derived from experiments using heterologously co-expressed GPCRs in HEK293T cells and no functional consequences were identified. Furthermore, due to the absence of negative controls, it can not be excluded that our results represents artifacts derived from potentially high GPCR expression levels which might force interactions that would not occur at physiological relevant expression levels. As such, the data presented in **Chapter 3** should be carefully interpreted and considered as a first indication that ORF74 forms heterodimers with human chemokine receptors. However, the successful application of the PLA technique offers opportunities to study ORF74 dimerization in a native environment to meet the criteria for GPCR dimerization set by a IUPHAR committee.

5. HIJACKING OF HUMAN RECEPTOR TYROSINE KINASES

GPCRs and receptor tyrosine kinases (RTKs) belong to different receptor classes and differ in their structure and activation mechanism. Yet, GPCRs and RTKs are more than 'just neighbors' on the cell surface as they can transactivate each other [313] and share several interaction partners.

The aim of **Chapter 4** was to investigate whether ORF74 crosstalks with the human insulin-like growth factor 1 receptor (IGF-1R), a RTK linked to Kaposi's sarcoma [327]. Our data (Fig. 5) show that:

- ORF74 is unable to transactivate IGF-1R.
- IGF-1 activates PLC signaling in cells expressing constitutive active ORF74, but not in mock- or US28-transfected cells. Trying to elucidate the underlying mechanism, we found that:
    - IGF-1 most likely does not bind directly to ORF74 but instead acts via IGF-1R, as shown by siRNA-mediated knockdown of IGF-1R and IGF-1R antibodies that inhibit IGF-1 binding.
    - IGF-1 is unable to activate PLC when co-stimulated with the inverse agonist of ORF74 CXCL10 or in cells expressing the functionally inactive mutant ORF74-$R^{3.50(143)}$A, suggesting that the constitutive activity of ORF74 is required for PLC activation in response to IGF-1
    - IGF-1 activates PLC in cells expressing a chemokine binding-deficient ORF74 mutant, showing that IGF-1R transactivates ORF74 independently of ORF74 chemokines.
    - Ala-substitution of tyrosine residues in the C-tail and ICL2 of ORF74 did not impair IGF-1-mediated PLC activation, ruling out a role for ORF74 tyrosine phosphorylation of these residues.
    - IGF-1R and OF74 are in close proximity, as determined by the proximity ligation assay

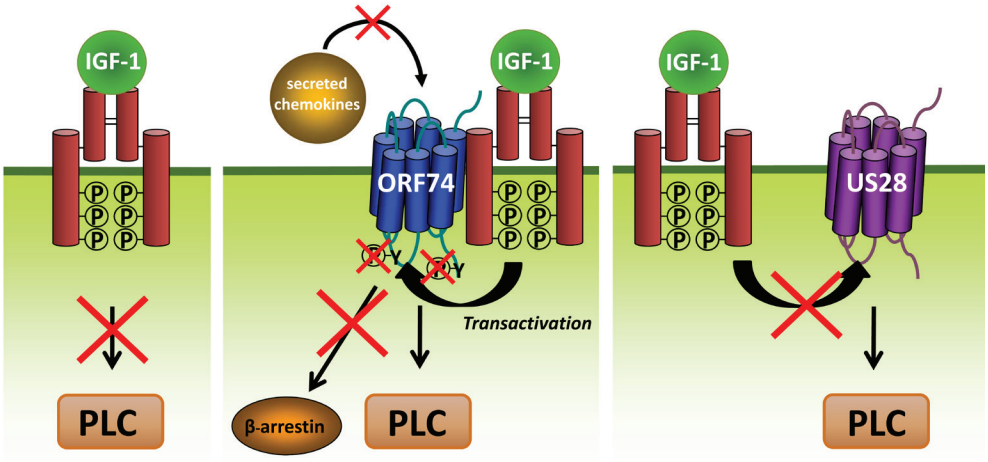

Figure 5. Crosstalk between IGF-1R and ORF74. *Schematic overview of the mechanism underlying IGF-1-induced PLC activation in mock-* ***(left)****, ORF74-* ***(middle)*** *or US28* ***(right)****-transfected HEK293T cells endogenously expressing IGF-1R.*

5.1 RTK Transactivation

Although ORF74 does not transactivate IGF-1R in HEK293T cells, it remains to be investigated whether ORF74 transactivates other RTKs. One potential candidate is the platelet-derived growth factor receptor (PDGFR), as ORF74 mediates secretion of its ligand PDGF [557]. Furthermore, the PDGFR inhibitor imatinib inhibits the survival of a Kaposi's sarcoma cell line [558] and administration to patients with AIDS-related Kaposi's sarcoma resulted in tumor regression [559], suggesting a role of PDGFR in Kaposi's sarcoma.

The epidermal growth factor receptor (EGFR) is transactivated by a growing list of GPCRs [560]. EGFR transactivation involves activation of matrix metalloproteases (MMPs), resulting in the ectodomain shedding of a EGF-like ligand that subsequently activates EGFR. The mechanism was recently further elucidated by showing that G proteins are able to directly activate MMP14 [561]. On the other hand, β-arrestin is required for β_1AR- and Urotensin receptor (UTR)-mediated transactivation of EGFR in vitro and in vivo, as shown by siRNA-mediated knockdown of β-arrestin1/2 and β-arrestin2 knockout mice [562, 563]. Furthermore, EGFR can be transactivated by kinases such as Src [564]. Transactivation of EGFR has been linked to cancer. A broad analysis revealed that EGFR transactivation in response to GPCR ligands occurs in more than 60 human carcinoma cell lines (e.g. prostate carcinoma, head and neck squamous cell carcinoma, colon cancer, ovarian carcinoma, glioblastoma and lung carcinoma) and leads to cell proliferation, migration and anti-apoptosis [565, 566]. Although a role for EGFR in Kaposi's sarcoma has not (yet) been established, it might be interesting to investigate whether EGFR is transactivated by ORF74 as ORF74 activates proteins involved in EGFR transactivation such as MMP2 and Src [567].

5.2 GPCR transactivation; the mechanism underlying the ORF74 /IGF-1R crosstalk

Only a handful of GPCRs have been shown to be transactivated by IGF-1R (e.g. CXCR4 [321],

CCR5 [317], the pituitary adenylyl cyclase activating polypeptide type 1 receptor (PAC1R) [322] and S1P1R [320]) and the mechanism behind this crosstalk is often not completely understood. We show that this crosstalk is not restricted to human GPCRs as ORF74 is also transactivated by IGF-1R. Although we excluded several possible mechanism, the exact mechanism underlying the ORF74/IGF-1R crosstalk remains to be elucidated.

5.2.1 Interaction between ORF74 and IGF-1R

IGF-1R and ORF74 are in close proximity as determined by the proximity ligation assay (Fig. 5) and this potential interaction might allow transactivation across the ORF74/IGF-1R interaction interface in response to IGF-1. A constitutive physical interaction was also observed between IGF-1R/CXCR4 [321], IGF-1R/PAC1R [322], IGF-1R/$GABA_B$ receptor [316], PDGF/S1P1R [324] and the Trk A receptor/lysophosphatidate 1 receptor (LPA1R) [568]. However, it remains to be elucidated if these observed interactions are required for the observed crosstalk. Similar to what was discussed for GPCR dimers (see section 4.1), disruption of the GPCR/RTK complex would provide direct evidence but is also challenging. Knowledge about the contact interface is required and preservation of the individual signaling pathways is needed to draw the right conclusions. For example, Lin et al. developed an activity-based probe to study the dynamics of a $GABA_B$ receptor/IGF-1R signaling complex [413]. Activation of the $GABA_B$ receptor leads to the dissociation of preassembled $G\alpha_i$ and $G\beta$ proteins from the $GABA_B$ receptor and association of IGF-1R and downstream signaling proteins. The G_i inhibitor PTX perturbed these dynamic interactions and subsequently inhibited transactivation of IGF-1R, suggesting that the dynamics of this protein complex are critical for transactivation [413]. However, as PTX inhibits proteins that act downstream of the $GABA_B$ receptor, it is difficult to directly link the disrupted protein complex to the inhibited transactivation of IGF-1R. In another study, the synergistic activation of ERK upon co-activation of the adenosine 2A receptor ($A_{2A}R$) and the fibroblast growth factor receptor (FGFR) was attributed to the interaction between both receptors, as a synthetic peptide corresponding to the interaction interface of $A_{2A}R$ with FGFR disrupted the $A_{2A}R$/FGFR interaction and the synergistic activation of ERK [569]. However, controls to exclude that the synthetic peptide directly alters the functionality of $A_{2A}R$ and FGFR were lacking.

5.2.2 Distinct ORF74 conformations

Interestingly, IGF-1 does not induce the recruitment of β-arrestins to ORF74 (Fig. 5). This might indicate that IGF-1 and CXCL1 induce different conformations of ORF74. Both conformations are able to activate PLC but only the CXCL1-induced conformation is able to recruit β-arrestins. Intramolecular FRET sensors might be used to study these possible distinct ORF74 conformations and have previously been described for the bradykinin B_2 receptor [570], $A_{2A}R$ [571], α_{2A}-adrenergic receptor [572] and PTHR [572]. In these sensors, cyan fluorescent protein (CFP) is fused in frame with the C-tail of the receptor and yellow fluorescent protein (YFP) is incorporated into ICL3 (or vice versa). Rearrangement of the TM helices upon receptor activation is measured as a change in energy transfer between CFP and YFP and was used to reveal distinct agonist-specific conformation of $A_{2A}R$ [573]. More recently, YFP has been replaced by the fluorescein arsenical hairpin (FlAsH) binding sequence to which binding of a non-fluorescent probe generates fluorescence [571, 573-575]. As FlAsH is much smaller than CFP, the risk of perturbing the overall structure of the receptor is reduced. However, the ICL3 of ORF74 (8 residues) is much smaller as compared

to other FlAsH-tagged GPCRs (for example, 156 residues for the α_{2A}R [576]) and it remains to be investigated whether a FlAsH sequence would be tolerated in ORF74.

5.2.3 Tyrosine phosphorylation of G proteins

Another explanation for the inability of IGF-1 to induce β-arrestin recruitment to ORF74 is that the observed crosstalk occurs downstream of ORF74. IGF-1R or a tyrosine kinases acting downstream of IGF-1R (e.g. Src) might phosphorylate the Gα subunit that is released from the heterotrimeric G protein complex upon activation of ORF74. This might explain why IGF-1 is unable to activate PLC in cells expressing the functionally inactive mutant ORF74-$R^{3.50(143)}$A or in the presence of the inverse agonist CXCL10. Indeed, tyrosine phosphorylation of G proteins ($G\alpha_q$, $G\alpha_i$ and $G\alpha_s$) in response to growth factors contributes to enhanced G protein activation [577-580]. Furthermore, stimulation of G_q-coupled GPCRs (e.g. metabotropic glutamate receptor 1α (mGluR1α) and M_1 mAChR) results in the phosphorylation of Y_{356} of $G\alpha_q$. PLC activation in response to stimulated receptors was blocked by protein tyrosine kinase inhibitors or by the co-expression of a tyrosine phosphorylation-deficient mutant of $G\alpha_q$ [581].

Possibly, this mechanism requires the close proximity between IGF-1R and ORF74, enabling the immediate accessibility of the Gα subunit for tyrosine phosphorylation by IGF-1R. A similar mechanism was proposed to underlie the crosstalk between PDGF and S1P1R [323]. PDGF and S1P1R form a complex, which was claimed to facilitate PDGF-induced tyrosine phosphorylation of $G\alpha_i$ and the subsequent activation of ERK1/2 [323]. As no close proximity was observed between US28 and IGF-1R using PLA, this might be the reason why IGF-1 is unable to increase basal PLC activation of US28. Another explanation is that US28 and ORF74 might use different G proteins for PLC activation. US28 activates PLC in a $G\alpha_q$-dependent manner, as was shown by using $G\alpha_q$-deficient MEF cells [285]. However, such experiments were not performed for ORF74 and it remains to be elucidated whether ORF74 also uses $G\alpha_q$ to activate PLC (see also section 2).

5.3 GPCRs and RTKs share signaling proteins

G proteins, GRKs and β-arrestins are proteins typically involved in GPCR signaling. RTKs interact with proteins such as Grb2, Shc and PLCγ to activate downstream signaling. However, it is becoming more and more apparent that RTKs behave as 'GPCR like receptors' by utilizing GPCR interaction partners such as G proteins. For example, $G\alpha_i$ was co-immunoprecipitated with IGF-1R [582-584] and IGF-1 stimulation leads to GTP loading of $G\alpha_{i2}$ [585]. Furthermore, IGF-1-induced ERK1/2 activation and Ca^{2+} mobilization is sensitive to PTX [447, 583, 586] or sequestration of Gβγ [586]. Other RTKs, such as EGFR and FGFR also interact with G proteins [587]. However, it is unknown whether RTKs directly interact with G proteins and act as guanine nucleotide exchange factors to activate G proteins or whether this is mediated by an associated and/or transactivated GPCR. On the other hand, $G\alpha_{13}$ is required for PDGF-, EGF- and vascular endothelial growth factor (VEGF)-induced cell migration as shown by siRNA-mediated knockdown of $G\alpha_{13}$ or MEF cells derived from $G\alpha_{13}^{-/-}$ mouse embryos. Interestingly, a GPCR-coupling defective $G\alpha_{13}$ mutant could rescue the migratory responses of PDGF and EGF in $G\alpha_{13}$ knockout cells, suggesting that growth factor-mediated migration through $G\alpha_{13}$ is independent of GPCRs [588].

In a similar fashion, IGF-1R, insulin receptor (IR) and EGFR co-immunoprecipitate with β-arrestins in a ligand-dependent manner [589]. β-arrestins regulate the internalization and ERK activation of IGF-1R [590] and GRK2, GRK5 and GRK6 play a key role in these event [420]. Ala-substitution of two serine residues in the intracellular domain of IGF-1R abrogated β-arrestin recruitment in response to IGF-1 [420], providing strong evidence for a direct interaction of IGF-1R with β-arrestins. However, it remains to be elucidated how β-arrestin (see section 3.1.2) recognizes RTKs that lack a 7TM core domain.

Reciprocally, a few examples show that GPCRs are also able to interact with typical RTK interaction partners. AT_1R binds to PLCγ in response to its ligand AngII in a tyrosine phosphorylation-dependent manner [591]. Furthermore, the C-tail of β_2AR is phosphorylated on Y^{350} in response to insulin and IGF-1 and subsequently binds Grb2. As a consequence, Grb2 links β_2AR to dynamin, resulting in insulin- and IGF-1-induced internalization of β_2AR and ERK activation [592]. β_2AR-$Y^{350}F$ fails to bind Gbr2 and subsequently fails to internalize in response to insulin [592, 593].

6. RELEVANT CELL LINES AS TOOLS TO STUDY ORF74 SIGNALING, DIMERIZATION AND CROSSTALK

To identify potential ORF74 signaling mechanisms, we initially used HEK293T cells due to their high transfection efficiency [594], which is necessary for the functional analysis of receptor mutants and fusion proteins. However, confirming our findings in (patho)-physiologically relevant cells is an important next step as GPCR/RTK crosstalk and signaling might be cell type-dependent [94, 595]. Several cell lines susceptible for KSHV infection or derived from KSHV-associated tumors have been used to study ORF74 signaling (Table 1). For example, three different cell lines have been derived from Kaposi's sarcoma named Y-1, SLK and KSIMM [83, 546]. However, Y-1 cells turned out to be indistinguishable from T24 urinary bladder cancer cell line and short tandem repeat profiling revealed that the SLK cell line is an contaminant of the renal cell carcinoma cell line Caki-1 [596]. Other relevant cell lines to study ORF74 signaling are endothelial cells, monocytes and B-cells such as PEL-derived cell lines (see Table 1). Most PEL cell lines are latently infected with KSHV and need to be treated with tetradecanoyl phorbol acetate (TPA), phorbol 12-myristate 13-acetate (PMA) or sodium butyrate to activate the KSHV lytic cycle in order for ORF74 to be expressed. siRNA-mediated depletion of ORF74 is often used to study the specific role of ORF74 [498, 597]. Another example to study ORF74 signaling in a viral context is to infect cells with KSHV [64] or to transfect cells with bacterial artificial chromosomes (BAC) containing the HHV-8 genome, which allows for mutagenesis of ORF74 [597, 598].

7. ORF74 AS A DRUG TARGET

ORF74 activates proliferative, pro-inflammatory and angiogenic pathways via autocrine and paracrine mechanisms that have been correlated with the onset and progression of KS ([53, 155]). Mutagenesis studies have shown that both constitutive activity and ligand-induced signaling are required for the development of KS-like lesions in transgenic mice

Table 1. (Patho)-physiologically relevant cells to study ORF74 signaling, dimerization and crosstalk

Cells			ORF74 expression	ORF74 signaling	References
B cells	Primary B cells from KS patients			NF-κB	[189]
	PEL-derived cell lines	BC3	PMA-induced KSHV lytic replication or stable ORF74 expression under control of an inducible promoter	ERK, p38, AP-1, CREB, NF-κB, NFAT, ORF50, ORF57, VEGF, survival	[179, 189, 497, 498]
		JSC-1	TPA-induced KSHV lytic replication or expression of ORF50	ORF74 expression regulated by KSHV-encoded K7	[337, 528]
		BCBL-1	TPA-induced KSHV lytic replication	ORF50, ORF74 expression regulated by KSHV-encoded K7	[498, 528]
Endothelial cells		DMVEC	retroviral transduction	NF-κB	[83]
		HUVEC	retroviral transduction, transient transfection (Fugene), stable transfection (Hiperfect)	VEGF, Akt, survival, IL-6, IL-8, and CCL5, SWAP70	[83, 185, 326, 607]
		Human pulmonary arterial endothelial cells	retroviral transduction	MMP2, Src	[567]
		TIME cells	infection with BCBL-1-derived KSHV followed by TPA treatment	viral replication	[597]
		Mouse lung endothelial cells	stable transfection (Lipofectin)	PLC, cAMP inhibition, PI3K, NF-κB	[608]
		mouse SVEC	stable transfection (Hiperfect, ExGen 500, Fugene)	SWAP70, tumor formation, NF-κB vascular permeability, Akt, mTor	[607, 609-611]
	KS-derived cells	KSIMM	transient transfection (Fugene)	NF-κB, IL-6, IL-8, and CCL5, VCAM-1, ICAM-1, E-selectin	[597]
Monocytes		THP-1 cell line	transient transfection (Effectene)	NF-κB, IL-1β, IL-6, TNF-α	[190]

[155]. Although the majority of KSHV-infected KS cells do not express ORF74, discontinuing ORF74 expression in an inducible transgenic mouse model or selectively eliminating the few ORF74-expressing cells in established tumors in an allograft mouse model resulted in a reduced expression of angiogenic factors, apoptosis of surrounding cells that do not express ORF74 and subsequent regression of KS-like lesions [84, 329]. Hence, inverse agonists that target the constitutive activity and chemokine binding to ORF74 might represents attractive therapeutics.

7.1 Targeting the non-canonical pathways

How do the findings described in this thesis contribute to the targeting of ORF74? β-arrestin recruitment and internalization of ORF74 suggest that it is possible that ORF74 signals from intracellular compartments. Although intracellular signaling of ORF74 has not yet been investigated, inverse agonists that also inhibit internalization might be beneficial if intracellular ORF74 signaling contributes to KS. On the other hand, if internalization mainly terminates G protein signaling of ORF74, ligands that promote internalization without activating ligand-induced oncogenic G protein-dependent signaling might be of interest if inverse agonists are unavailable. Such biased ligands that selectively block undesired responses but promote beneficial effects have been described for several GPCRs [599]. For example, TRV120027 antagonizes G protein-mediated signaling of AT1R that has been linked to hypertension while it promotes β-arrestin recruitment, internalization and β-arrestin-dependent signaling that elicit beneficial responses for cardiac performance [600].

In addition, ORF74 internalization could be exploited for the selective uptake of drugs. Previously, liposomes containing surface-bound polyethylene glycol (PEG) were used as carriers for the cytotoxic agent doxorubicin to treat KS patients [601]. PEGylated liposomes are passively targeted to tumor vasculature, but often do not accumulate inside tumor cells [602]. Immobilization of ORF74 agonists to nanoparticles might be a strategy for the ORF74-mediated internalization of these particles and the subsequent targeting of cytotoxic drugs into ORF74-expressing KS cells [603]. This delivery approach has successfully been applied by targeting several GPCRs, including somatostatin receptors, cholecystokinin receptors, luteinizing hormalone-releasing hormone (LHRH) receptors, neurotensin receptors and the neuropeptide Y1 receptor [604, 605].

Studies with ORF74 mutants indicate that the modulation of constitutive ORF74 signaling is essential for the development of KS-like tumors in transgenic mice [155]. Although the pathological relevance of the crosstalk between IGF-1/IGF-1R and ORF74 needs to be elucidated in vivo, the observed modulatory effects of IGF-1 on constitutive ORF74 signaling might offer opportunities to target RTKs as a KS treatment. However, this approach would not block constitutive and chemokine-induced ORF74 signaling and a combined treatment by antagonizing the modulatory activity of both CXCL1 and IGF-1 might be required. On the other hand, blocking RTK function might be of therapeutic benefit if RTKs act downstream of ORF74 (see section 5.1).

7.2 Non-specific mechanisms; pitfalls for identifying small-molecule inhibitors (and activators)

In **Chapter 5**, we aimed at identifying the first small-molecule inhibitors for ORF74. A

structure-based virtual screening was performed, using a CXCR4-based homology model of ORF74. This resulted in the identification of 5 compounds that inhibited ^{125}I-CXCL8 binding to ORF74 and ORF74-induced PLC activation. However, these compounds also inhibited PLC activation in response to AlF_4^- in cells that did not express ORF74. As such, we cannot exclude that the identified compounds show off-target effects as well.

Screening programs often suffer from false positives that non-specifically inhibit (or activate) target proteins [436]. Several mechanisms have been proposed to underlie the action of false positives. Compounds may chemically alter proteins in a non-specific way, produce molecules with oxidizing and/or reducing properties that inhibit the target protein or show cytotoxicity. Other examples involve compounds that sequester metal ions that are required for the functionality of the target protein, compounds that have spectral properties that interfere with the assay read-out [436] or form aggregates that may interact and subsequently inhibit proteins [486]. At concentrations that we used to identify compounds that inhibit ORF74-mediated PLC activity (30 uM), 19% of drug-like molecules can form aggregates [487].

In some publications the identified hits were tested to form aggregates. For example, Mysinger and coworkers removed putative aggregates by centrifugation and counterscreened their target protein with the supernatant. Others have made use of the detergent-sensitive nature of aggregated compounds. Inhibition of the target protein in the absence but not in the presence of detergent is characteristic for aggregating compounds. Alternatively, dynamic light-scattering (DLS) allows for the direct measurement of aggregation [437, 486, 606]. However, these techniques do not exclude a role for other non-specific mechanisms. Furthermore, counterscreening for cytotoxicity or aggregation is not even included in most publications. Interestingly, many journals have strict policies with regard to the purity and identity of compounds, but not many require evidence to exclude non-specific mechanisms. In January 2015, the journal of Medicinal Chemistry revised their guidelines and authors are now obligated to provide evidence from at least two different assays to show that the apparent activity of compounds is not an artifact (e.g. by interfering with assay read-out). In my opinion, testing for non-specific mechanism should always be incorporated in screening programs and should be included in the guidelines of all journals. For inhibitors, the best way to do this is to counterscreen potential hits for inhibiting signaling that is activated independently of the GPCR. For example, the GPCR of interest can be bypassed by chemically activating signaling (e.g. by G protein activators such as AlF_4^-, as used in **Chapter 5**), by expressing constitutive G proteins or expressing an unrelated GPCR that activates the same pathway.

8. CONCLUDING REMARKS

Taken together, we have shown that ORF74 potentially modulates cellular signaling via non-canonical pathways. ORF74 hijacks human trafficking routes by using mechanisms that are also employed by many human chemokine receptors. Furthermore, we provide a first indication that ORF74 interacts with human chemokine receptors potentially to modulate the

latter in favor of the virus. Finally, we have shown that ORF74 unmasks signaling of IGF-1 to PLC. Future studies need to confirm these results in (patho)physiologically relevant KSHV-positive cells. The results in this thesis show that modulating cellular signaling by ORF74 is not restricted to canonical mechanism. It remains to be investigated how the non-canonical pathways contribute to immune evasion, viral dissemination and KSHV-associated pathologies and if they are generally used by HHVs to modulate cellular signaling.